Methotrexate and vasculoprotection: mechanistic insights and potential therapeutic applications in old age

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Abstract

Background: Increasing age is a strong, independent, risk factor for atherosclerosis and cardiovascular disease. Key abnormalities driving cardiovascular risk in old age include endothelial dysfunction, increased arterial stiffness and blood pressure, and the pro-atherosclerotic effects of chronic, low-grade, inflammation. The identification of novel therapies that comprehensively target these alterations might lead to a major breakthrough in cardiovascular risk management in the older population. Systematic reviews and meta-analyses of observational studies have shown that methotrexate, a first-line synthetic disease-modifying anti-rheumatic drug, significantly reduces cardiovascular morbidity and mortality in patients with rheumatoid arthritis, a human model of systemic inflammation, premature atherosclerosis, and vascular ageing.

Methods and results: We reviewed the *in vitro* and *in vivo* studies investigating the effects of methotrexate on endothelial function, arterial stiffness, and blood pressure, and the potential mechanisms of action involved. The available evidence suggests that methotrexate might exert beneficial effects on vascular homeostasis and blood pressure control by targeting specific inflammatory pathways, adenosine metabolism, and 5' adenosine monophosphate-activated protein kinase. Such effects might be biologically and clinically relevant not only in patients with rheumatoid arthritis but also in older adults at high cardiovascular risk.

Conclusions: Methotrexate has the potential to be repurposed for cardiovascular risk management in old age in view of its putative pharmacological effects on inflammation, vascular homeostasis, and blood pressure. However, the further study and confirmation of these effects are essential in order to adequately design intervention studies of methotrexate in the older population.

Key words: methotrexate, cardiovascular risk, ageing, atherosclerosis, endothelium, arterial stiffness, blood pressure, inflammation.

1. INTRODUCTION

Atherosclerotic cardiovascular disease remains the leading cause of death worldwide in spite of significant advances in diagnosis, treatment, and prevention [1]. Two clinical manifestations of atherosclerotic cardiovascular disease, ischaemic heart disease and cerebrovascular disease, account for about 85% of all cardiovascular disease deaths [1]. Cardiovascular disease primarily affects the older population because of the lifelong accumulation of risk factors and the development of significant functional and structural alterations of the arterial wall that favour the development of atherosclerosis and thrombosis in this group [2-4]. Experimental and clinical evidence has also highlighted the important pathophysiological role of inflammation, driven by specific pro-atherosclerotic cytokines, in favouring vascular damage and atherosclerosis [4, 5]. The role of inflammation is further supported by the recent identification of a new cardiovascular risk paradigm, residual inflammatory risk. This describes a relatively large group, between 29-47% of patients with atherosclerotic cardiovascular disease, with persistently high inflammatory risk markers and cardiovascular risk despite maximal treatment with cardioprotective drugs, particularly statins [6]. However, available cardiovascular drugs do not specifically target pro-atherosclerotic inflammatory pathways. The identification of new agents with anti-inflammatory and vasculoprotective effects might significantly improve primary and secondary cardiovascular prevention, particularly in patients with residual inflammatory risk [6-10]. While a significant amount of research is focused on the discovery of such agents the study of human models of premature atherosclerosis and vascular ageing, in the context of a chronic pro-inflammatory state, might lead to the identification of previously unknown vasculoprotective effects of currently available anti-inflammatory and immunomodulating drugs. Repurposing these drugs for cardiovascular risk management would circumvent the need for time-consuming, highly-

expensive drug discovery and development programs, therefore facilitating their routine use in clinical practice [11].

This review discusses the vascular alterations associated with ageing, and their similarities with those reported in rheumatoid arthritis, an autoimmune condition characterized by chronic systemic inflammation and high cardiovascular risk. It also discusses the epidemiological and clinical studies investigating the association between the use of the first-line disease modifying anti-rheumatic drug (DMARD) methotrexate and cardiovascular risk, the *in vitro* and *in vivo* studies on the vasculoprotective effects of methotrexate and the mechanisms involved, and the potential repurposing of this drug for cardiovascular risk management, particularly in the older patient population.

2. VASCULAR AGEING: PATHOPHYSIOLOGY AND CLINICAL IMPLICATIONS

The vascular alterations occurring with advancing age primarily consist of endothelial dysfunction and an increase in arterial stiffness and blood pressure in the context of a chronic, low-grade, non-specific pro-inflammatory state (Figure 1).

2.1 Endothelial dysfunction

The endothelium, primarily through the synthesis of the key messenger nitric oxide (NO) by the enzyme endothelial NO synthase (eNOS), plays a key role in the modulation of arterial stiffness, peripheral vascular resistance, and blood pressure. This is supported by experimental and human studies showing that the pharmacological inhibition of eNOS causes a significant increase in arterial stiffness, peripheral vascular resistance, and blood pressure [12-14]. The endothelium also exerts atheroprotective effects by preventing leukocyte adhesion, vascular smooth muscle cell hypertrophy and proliferation, and platelet aggregation

[15-17]. There is good evidence that advancing age *per se* is associated with reduced synthesis and/or increased degradation of endothelial NO [18, 19]. One possible reason for the impaired synthesis of NO is the reduced availability and/or oxidation of the critical eNOS cofactor tetrahydrobiopterin. This leads to the synthesis of superoxide, instead of NO, by "uncoupled" eNOS [20]. This phenomenon has been observed in experimental models of ageing [21, 22]. Another factor potentially involved in the reduced synthesis of NO with advancing age is represented by an increased activity of arginase, an enzyme that competes with eNOS for the substrate L-arginine [23]. Several studies have also investigated the factors responsible for the increased degradation of NO associated with ageing. In particular, the presence of high local concentrations of reactive oxygen species (ROS), in the context of chronic inflammation and stimulation of the cytokine tumour necrosis factor (TNF)-α, and the activation of the renin-angiotensin-aldosterone system, are likely to favour excessive NO degradation [24-26]. In addition to the dysregulation of NO metabolic pathways, an imbalance between other endogenous vasodilators and vasoconstrictors, favouring vasoconstriction, arterial stiffening, and blood pressure elevation, has also been reported with advancing age. In particular, increasing concentrations of the endogenous vasoconstrictors endothelin-1, prostaglandin H_2, and thromboxane A_2, have been observed in experimental and human ageing [27-30]. Furthermore, an impairment of the vasodilating effects of prostacyclin has been reported with advancing age [31].

High ROS concentrations can also favour endothelial cell senescence, a phenomenon characterized by the interruption of the cell cycle and specific phenotypic changes [32]. These alterations cause a further increase in the synthesis and release of inflammatory cytokines, mediated by the activation of specific pathways such as the nuclear factor NF-κB, p38 mitogen-activated protein kinases, the DNA damage response pathway, and the transcription factor GATA-4 [33, 34]. Therefore, endothelial cell senescence significantly contributes to

the pro-inflammatory state of the arterial wall and the development of vascular damage and atherosclerosis [35, 36].

2.2 Increased arterial stiffness and blood pressure

The strong and independent association between advancing age and increased stiffness of the large conduit arteries, particularly the thoracic and abdominal aorta, is well documented [37]. The primary structural abnormalities underlying this phenomenon are represented by a reduction in elastic fibres and a concomitant increase in the amount of collagen in the arterial wall [38]. The accumulation of cardiovascular risk factors with ageing, particularly hypertension, has been proposed as the primary mechanism responsible for the increase in arterial stiffness, through an impairment in endothelial function and NO synthesis and an increase in sympathetic nervous system activity [39-44]. An additional factor that likely contributes to the increase in arterial stiffness with ageing is represented by a systemic pro-inflammatory state [45]. Although the exact mechanisms responsible for the inflammation-induced increase in arterial stiffness are unclear, the detrimental effect of specific inflammatory pathways on endothelial NO synthesis and the concomitant dysregulation of other pathways, such as the mechanistic target of rapamycin, the 5' adenosine monophosphate-activated protein kinase (AMPK), and sirtuins, might play a role [46-50].

An increase in large artery stiffness causes a further increase in systolic blood pressure and a reduction in diastolic blood pressure, with a consequent increase in pulse pressure [51]. Markers of increased arterial stiffness, such as the augmentation index and pulse-wave velocity, and higher systolic and pulse pressure values increase cardiac afterload and independently predict cardiovascular morbidity and mortality in middle-age and older adults [52-55]. At the same time, a reduction in diastolic blood pressure might critically impair coronary perfusion, with the consequent risk of myocardial ischaemia and adverse cardiovascular outcomes [56, 57].

3. RHEUMATOID ARTHRITIS: A HUMAN MODEL OF PREMATURE VASCULAR AGEING AND ATHEROSCLEROSIS

Rheumatoid arthritis is a chronic disabling autoimmune condition that is characterized by chronic local and systemic inflammation, joint pain, stiffness, and fatigue. Patients with rheumatoid arthritis also suffer from so-called "extra-articular" manifestations that affect several organs and systems and further contribute to disability and poor quality of life [58]. The disease-modifying anti-rheumatic drugs (DMARDs), cornerstone of treatment in rheumatoid arthritis, suppress the immune system, reduce local and systemic inflammation, and maintain physical and functional independence [58].

Rheumatoid arthritis is associated with higher mortality compared to the general population [59]. The excess mortality in rheumatoid arthritis is primarily due to cardiovascular death, with a standardized mortality ratio of 1.46 compared to the general population [60]. It is postulated that the state of chronic systemic inflammation in rheumatoid arthritis, and the activation of pro-atherosclerotic cytokines such as TNF-α, interleukin 1 (IL-1), and interleukin-6 (IL-6), favours endothelial dysfunction, vascular damage and atherosclerosis (Figure 1) [61-64]. However, traditional risk factors, such as hypertension, diabetes, obesity, and hypercholesterolaemia, also contribute to the pathogenesis of vascular dysfunction and the risk of adverse cardiovascular outcomes in this group [65].

Endothelial dysfunction is common in rheumatoid arthritis and affects both macrovascular and microvascular beds [66, 67]. In particular, microvascular endothelial dysfunction affects approximately 33% of patients even in the absence of overt cardiovascular disease [61]. This might account for the reduced coronary flow reserve, and consequent increased risk of ischaemic heart disease, reported in patients with rheumatoid arthritis and other similar

autoimmune conditions [68]. In addition to the potential role of inflammatory pathways and traditional cardiovascular risk factors, the accumulation of the potent endogenous inhibitor of eNOS synthase, asymmetric dimethylarginine (ADMA), might also play a role in the pathogenesis of endothelial dysfunction in rheumatoid arthritis [69-71].

The presence of endothelial dysfunction, with the consequent impairment in NO synthesis, leads to an increase in arterial stiffness in patients with rheumatoid arthritis [72]. Age, disease duration and severity, rheumatoid factor status, systolic blood pressure, leukocyte count, cholesterol fractions, and use of TNF-α inhibitors have shown independent associations with arterial stiffness in this group [73-75]. This suggests, similar to endothelial dysfunction, a combined contribution of specific disease markers, inflammatory markers and traditional cardiovascular risk factors to the pathogenesis of arterial stiffening in rheumatoid arthritis. An increase in arterial stiffness in rheumatoid arthritis exerts detrimental effects on blood pressure, cardiac afterload, and clinical outcomes that are similar to those described with ageing [76-78]. Studies on the relationship between rheumatoid arthritis and the incidence and prevalence of hypertension, when compared to the general population, have provided conflicting results [79]. However, recent evidence suggests sub-optimal diagnosis and undertreatment of hypertension in this group, with consequent adverse effects on arterial structure and function [80-82].

4. METHOTREXATE AND CARDIOVASCULAR DISEASE

Methotrexate is a relatively old, first-line, DMARD for the treatment of rheumatoid arthritis [83]. Notably, it is the only synthetic DMARD that has been shown to significantly reduce cardiovascular and all-cause mortality in this group [84]. Several studies have investigated the associations between methotrexate treatment and cardiovascular risk, endothelial function,

arterial stiffness, and blood pressure, in patients with and without rheumatoid arthritis or other autoimmune disorders.

4.1 Methotrexate and cardiovascular risk

Two systematic reviews and meta-analyses of observational studies in patients with rheumatoid arthritis and other autoimmune disorders have reported a significant reduction in cardiovascular events with methotrexate. The first meta-analysis of 10 studies in 66,334 patients showed that methotrexate use was associated with a significant reduction in total cardiovascular events (risk ratio, RR, 0.79, 95% CI 0.73 to 0.87) [85]. Similarly, the second meta-analysis of eight studies in 65,736 patients reported that the use of methotrexate was associated with a significant reduction in total cardiovascular events (RR 0.72, 95% CI 0.57 to 0.91) [86]. Following the publication of these meta-analyses, a population-based retrospective study investigated the association between methotrexate treatment and risk of ischaemic stroke in rheumatoid arthritis (n=7,904). The use of methotrexate was associated with a lower risk of ischaemic stroke during the first seven years of follow-up (adjusted hazard ratio, HR, 0.33, 95% CI 0.17 to 0.63). However, it was also associated with a higher risk of ischaemic stroke during the following three years (adjusted HR 3.36, 95% CI 1.70 to 6.61) [87]. A more recent observational study has specifically investigated the associations between methotrexate use and risk of cardiovascular events in 23,994 older patients with rheumatoid arthritis diagnosed after the age of 65 years. The use of methotrexate both in the previous 12 months and during the first year of follow-up was associated with a significant reduction in cardiovascular risk (HR, 0.79, 95% CI 0.70 to 0.88; and HR 0.84, 95% CI 0.72 to 0.96, respectively). However, a longer methotrexate exposure did not have a significant effect on cardiovascular risk (HR 0.98, 95% CI 0.95 to 1.01) [88].

In contrast with the overall results of observational studies, methotrexate treatment failed to show significant cardioprotective effects in a recent multicentre randomized placebo-

controlled trial in patients with high cardiovascular risk but without autoimmune disorders. In this study, 4,786 patients with a previous history of myocardial infarction or multivessel coronary artery disease and concomitant type 2 diabetes or metabolic syndrome were randomized to methotrexate treatment (target weekly dose 15-20mg) or matching placebo. After a median follow-up period of 2.3 years, methotrexate treatment was not associated with a significant reduction in a composite end-point non-fatal myocardial infarction, non-fatal stroke, or cardiovascular death (HR 0.96, 95% CI 0.79 to 1.16) [89]. The results of this study need to be interpreted with caution as both patients randomized to methotrexate and those randomized to placebo received oral folic acid 1mg daily for the duration of the study [89]. Oral folic acid treatment has been shown to enhance endothelial function and reduce blood pressure and arterial stiffness in human studies [90-92]. These effects might have translated into a beneficial effect on cardiovascular risk, as recently documented in other studies [93, 94]. Therefore, the administration of folic acid in the placebo group might have diluted the potential beneficial effects of methotrexate on cardiovascular risk in the active group.

4.2 Methotrexate, endothelial function, arterial stiffness, and blood pressure

Animal studies have shown conflicting results on the effects of methotrexate on endothelial function. Some studies reported negative effects [95, 96], whereas others described a significant improvement in endothelial function [97, 98]. However, the doses of methotrexate administered in these studies, between 24 and 490mg/day, are considerably higher than the doses typically prescribed in patients with autoimmune disorders, between 1 and 4mg/day. In human studies, methotrexate, singly or combined with other DMARDs, has been shown to enhance endothelial function in patients with inflammatory arthritis and rheumatoid arthritis [99-101].

In a study in patients with rheumatoid arthritis, methotrexate treatment did not reduce arterial stiffness [102]. By contrast, in a repeated cross-sectional study of patients with rheumatoid

arthritis, methotrexate treatment was independently associated with reduced pulse-wave velocity, a marker of arterial stiffness, measured over 24 hours, after adjusting for age, gender, body mass index, rheumatoid arthritis disease severity and use of folic acid [103].

Observational cross-sectional and prospective studies in rheumatoid arthritis have also shown that methotrexate use is associated with lower systolic and diastolic blood pressure and reduced incidence of hypertension, when compared to other DMARDs or no treatment [103-108]. In one of these studies, the associations between methotrexate treatment and lower blood pressure were also observed for 24-hr measures of peripheral and central blood pressure [103]. Twenty-four-hour averages of systolic and diastolic blood pressure are significantly superior to clinical blood pressure in terms of cardiovascular risk stratification [109]. Furthermore, measures of central blood pressure can better explain, when compared to peripheral blood pressure, the effects of different blood pressure lowering strategies on cardiovascular end-points [110]. In other studies, methotrexate has been shown to prevent the temporal increase in blood pressure mediated by arterial stiffness in rheumatoid arthritis, a phenomenon also reported with advancing age [111-113].

Therefore, the results of human studies generally support the hypothesis that methotrexate might exert beneficial effects on endothelial function, arterial stiffness, and blood pressure in patients with rheumatoid arthritis. Given the described similarities in the pathophysiology of arterial wall dysfunction between rheumatoid arthritis and ageing, it is plausible that the putative vasculoprotective effects of methotrexate might also be biologically and clinically relevant in the older patient population. The following sections discuss the pharmacology of methotrexate and the possible mechanisms responsible for the vasculoprotective effects of this drug.

5. PUTATIVE MECHANISMS OF METHOTREXATE-MEDIATED VASCULOPROTECTION

The intracellular polyglutamate forms of methotrexate inhibit the enzymes dihydrofolate reductase, thymidylate synthase, and aminoimidazole carboxamide ribonucleotide (AICAR) transformylase (ATIC) [114]. In particular, ATIC inhibition, with the consequent accumulation of the substrate AICAR, leads to the inhibition of the enzymes adenosine deaminase and adenosine monophosphate deaminases, responsible for the catabolism of adenosine (Figure 2) [115]. Adenosine is an important extracellular signalling molecule, with a half-life of a few seconds, that exerts significant anti-inflammatory effects through the A_{2A} and A_3 receptors [116]. These effects are likely to mediate the musculoskeletal anti-inflammatory and immunomodulatory effects of methotrexate in rheumatoid arthritis and other autoimmune disorders. However, the anti-inflammatory actions of adenosine might also exert vasculoprotective effects by preventing the increase in vascular permeability and endothelial cell damage induced by inflammatory stimuli, inhibiting vascular smooth muscle cell proliferation and suppressing atherosclerosis in experimental models [117-120]. Furthermore, primarily through the A_{2A} receptors, adenosine favours the opening of the K_v and K_{ATP} channels in vascular smooth muscle cells, with consequent membrane hyperpolarization, relaxation and vasodilation, and also stimulates NO synthesis by endothelial cells [121-123]. Although the direct vasodilatory effects of adenosine might account for the reported blood pressure lowering effects of this messenger, central mechanisms are also involved in the hypotensive response [124-126]. The role of adenosine in the regulation of blood pressure and arterial stiffness is further demonstrated by human studies showing that the pharmacological inhibition of the adenosine receptors A_1 and A_{2A} causes an acute increase in both parameters [127, 128]. Adenosine also inhibits the activity of the sino-atrial node, with a consequent reduction in heart rate [129, 130]. This phenomenon,

together with the additional bradycardic effects of adenosine mediated by the central nervous system, might also account for a reduction in arterial stiffness given the established effect of increasing heart rate on arterial stiffness both in animal models and in humans [126, 131-133].

The accumulation of AICAR, resulting from methotrexate-induced inhibition of ATIC, activates AMPK (Figure 2) [134]. Both AICAR and AMPK have been shown to stimulate endothelial NO synthesis and reduce blood pressure in experimental models [135-139]. AMPK might also play an important role in the regulation of arterial stiffness. This is supported by studies showing that the deficiency of the ageing-suppressor gene klotho accelerates the increase of arterial stiffness during a high-fat diet in mice, through the inhibition of AMPK expression [140]. By contrast, stimulation of AMPK prevents the increase in arterial stiffness in klotho-deficient mice [141].

Recent studies also suggest that methotrexate, either directly or through AICAR accumulation and/or AMPK activation, can significantly reduce the expression and/or the concentrations of the pro-atherosclerotic cytokines, TNF-α, IL-1, and IL-6. These cytokines have been shown to play an important role in favouring endothelial dysfunction, vascular damage, and atherosclerosis both in rheumatoid arthritis and in models of experimental ageing [142-145].

Therefore, the available *in vitro* and *in vivo* evidence supports the hypothesis that methotrexate might exert a unique combination of vasculoprotective effects through the accumulation of adenosine and AICAR, stimulation of AMPK, and down-regulation of TNF-α, IL-1, and IL-6 (Figure 3). However, further experimental and human investigations are warranted to establish the exact role of these biomarkers in mediating the effects of methotrexate treatment on surrogate and clinical cardiovascular end-points in intervention studies.

6. METHOTREXATE AND VASCULAR PROTECTION IN OLD AGE: PRACTICAL CONSIDERATIONS

The routine use of methotrexate for cardiovascular risk management might represent an attractive treatment option in the older patient population for a number of reasons. Treatment with methotrexate is associated with gastroenterological, haematological, renal, neurological, pulmonary and mucocutaneous toxicity [146, 147]. However, with appropriate dosing and monitoring, serious adverse events are relatively infrequent both in observational studies and in randomized controlled trials, between 2.1-5.5% [148-150]. Furthermore, in a study that specifically investigated the safety of methotrexate in 33 older patients with rheumatoid arthritis (mean age 78.8 years) followed for two years, treatment was discontinued in two patients because of abnormal liver function tests and in other two patients because of gastrointestinal side effects. No serious adverse events were described in this study [151]. These data support the overall safety and tolerability of methotrexate treatment in older patients.

A potential advantage of methotrexate treatment, particularly in a patient group that is often exposed to the unwanted consequences of inappropriate polypharmacy and complex medications regimens, is the once-weekly administration. This less intensive dosing regimen might ensure treatment adherence, which remains a significant issue in the routine management of cardiovascular risk in older patients [152, 153]. Furthermore, the key role of polyglutamates in mediating the vasculoprotective effects of methotrexate, if demonstrated in longitudinal studies, might translate into a favourable pharmacokinetic profile, in terms of treatment efficacy and safety, given the relatively long half-life of these intracellular forms of the drug [154]. This would ensure a sustained effect of methotrexate on vascular homeostasis and blood pressure in case of occasional dose missing. Furthermore, it would minimize the

risk of acute post-dose orthostatic hypotension, a frequent and potentially serious side effect of cardiovascular and non-cardiovascular medications in the older population [155].

However, the hypothesis that methotrexate can be repurposed for the management of cardiovascular risk in older patients needs to be robustly investigated in appropriately designed intervention studies, using either placebo or other DMARDs as comparator, in patients with different degrees of vascular dysfunction, combinations of risk factors, and residual inflammatory cardiovascular risk. Such trials should investigate the short- and long-term effects of methotrexate on surrogate markers of vascular damage (e.g. endothelium-dependent vasodilation, arterial stiffness, and blood pressure) as well as "hard" clinical end-points (e.g. non-fatal myocardial infarction and stroke, cardiovascular mortality and all-cause mortality). The use of DMARDs or other anti-inflammatory agents as comparator is required to test whether methotrexate exerts vasculoprotective effects that are either independent of inflammation or are mediated by methotrexate-specific inflammatory targets. Additionally, intervention studies should assess whether the potential vasculoprotective effects of methotrexate are mediated by intracellular polyglutamates and/or genetic polymorphisms of methotrexate transporters and target enzymes [156].

7. CONCLUSIONS

Patients with rheumatoid arthritis exhibit premature vascular ageing and have an increased cardiovascular risk in the context of a chronic systemic pro-inflammatory state. The identification of effective vasculoprotective therapies in rheumatoid arthritis might lead to their additional use in the older patient population, the main group suffering from the burden of atherosclerotic cardiovascular disease. Observational studies in patients with rheumatoid arthritis have shown that the DMARD methotrexate can reduce cardiovascular morbidity and mortality. *In vitro* and *in vivo* studies suggest that methotrexate might exert a unique

combination of anti-inflammatory and vasculoprotective effects through the accumulation of adenosine and AICAR, stimulation of AMPK, and down-regulation of TNF-α, IL-1, and IL-6. However, adequately designed intervention studies are warranted to determine the exact role of methotrexate in cardiovascular risk management in the older patient population.

Figure legends

Figure 1: Mechanisms mediating the increased cardiovascular risk in rheumatoid arthritis and advancing age.

Figure 2: Intracellular effects of methotrexate. ATIC, aminoimidazole carboxamide ribonucleotide (AICAR) transformylase; FAICAR, 5-formamidoimidazole-4-carboxamide ribotide; IMP, inosine monophosphate; AMP, adenosine monophosphate; AMPK, 5' adenosine monophosphate-activated protein kinase; 5'-NT, 5'-nucleotidase; -, inhibition; +, activation.

Figure 3: Putative mechanism mediating the potential vasculoprotective effects of methotrexate. AICAR, aminoimidazole carboxamide ribonucleotide; AMPK, 5' adenosine monophosphate-activated protein kinase; TNF, tumour necrosis factor, IL, interleukin.

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